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The Effects of a Pulsed Electromagnetic Field on the Proliferation and Osteogenic Differentiation of Human Adipose-Derived Stem Cells

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Manuscript Preparation E
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Background: A low frequency pulsed electromagnetic field (PEMF) has been confirmed to play an important role in promoting the osteogenic differentiation of human bone marrow stem cells (BMSCs). Adipose-derived stem cells (ASCs) possess some attractive characteristics for clinical application compared to BMSCs, such as abundant stem cells from lipoaspirates, faster growth, less discomfort and morbidity during surgery. ASCs can become adipocytes, osteoblasts, chondrocytes, myocytes, neurocytes, and other cell types. Thus, ASCs might be a good alternative in clinical work involving treatment with PEMF.

Material/Methods: Human ASCs (hASCs)were divided into a control group (without PEMF exposure) and an experimental group (PEMF for two hours per day). We examined the effect of PEMF on promoting cell proliferation and osteogenic differentiation from several aspects: CCK-8 proliferation assay, RNA extraction, qRT-PCR detection, western blotting, and immunofluorescence staining experiments.

Results: PEMF could promote cell proliferation of human ASCs (hASCs) at an early stage as determined by CCK-8 assay. A specific intensity (1 mT) and frequency (50 Hz) of PEMF promoted osteogenic differentiation in hASCs in alkaline phosphatase (ALP) staining experiments. In addition, bone-related gene expression increased after two weeks of PEMF exposure, the protein expression of OPN, OCN, and RUNX-2 also increased after a longer period (three weeks) of PEMF treatment as determined by western blotting and immunofluorescence staining.

Conclusions: We found for the first time that PMEF has a role in stimulating cell proliferation of hASCs at an early period, subsequently promoting bone-related gene expression and inducing the expression of related proteins to stimulate osteogenic differentiation.

MeSH Keywords: Adult Stem Cells • Cell Proliferation • Electromagnetic Fields • Osteogenesis

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Background

Pulsed electromagnetic fields (PEMFs) have been widely used in orthopedics for at least three decades [1,2] and have been useful in enhancing bone repair in nonunion fractures and related bone-healing problems [3,4]. Several *in vivo* investigations have also demonstrated that PEMF stimulation could inhibit bone loss and improve bone quality in various osteopathic animals [5,6]. Its non-invasive nature, absence of infection, easy treatment, side effects, and other advantages have resulted in increased attention on PEMFs [7]. Treatment of osteoporosis can effectively improve the bone mineral density in these patients [8–10]. The stimulation of bone at the fracture site by the introduction of electromagnetic fields may be similar to the resulting stimulation from mechanical loading [11,12]. Li et al. [13] found a different field strength in osteoblast osteogenesis.

In recent years, several researchers have confirmed that PEMF has a role in promoting mesenchymal stem cell proliferation and osteogenic differentiation [6,14,15]. Cell proliferation is important for cell survival and the expression of various biological activities. Specific PEMF frequencies have also been reported to enhance human adipose-derived stromal cell/bone marrow stem cell (hASC/BMSC) proliferation and play a key role in the use of hSSCs/BMSCs for tissue engineering [16]. Thus, PEMF has a clear effect on promoting the proliferation of BMSCs.

Osteogenesis is a biological process with complex regulatory mechanisms that include the differentiation of progenitor cells from BMSCs into osteoblasts and the secretion and mineralization of bone matrix by osteoblasts to form bone tissue [17,18]. Esposito et al. [19], Zhou et al. [20], and Jansen et al. [21] found that applying PEMF to BMSCs plays a role in promoting osteoblast differentiation as the expression of osteogenic markers was increased. Schwartz et al. [22] and Okada et al. [23] found that PEMFs can improve the effect of bone morphogenetic protein-2 (BMP-2) on osteogenic differentiation of BMSCs. PEMFs and BMP-2 thus have a synergistic effect.

In addition to the bone marrow, other major sources of MSCs can be derived from the human body [24]. ASCs share similar immunomodulatory properties with BMSCs and have the advantages of being readily available and abundant [25]. Compared to other types of stem cells, ASCs have many major advantages. First, ASCs can be easily obtained from subcutaneous liposuction [26]. Second, ASCs have no ethical or political problems compared to embryonic stem cells because ASCs can be derived from autologous fat [27]. Third, it is important to underline that ASCs can survive in low-oxygen environments, making them optimal for cell therapies where oxygen supplied by the vascular network may be limited, such as in implant surgery procedures. In fact, under hypoxic conditions, ASCs can increase vascular endothelial growth factor (VEGF) secretion, stimulating the proper angiogenesis needed for bone formation [28]. These features make ASCs a more acceptable cell type for tissue and organ transplantation in regenerative medicine and clinical research [29].

The use of ASCs as a clinical tool has progressed significantly over the past decade. Mesimaki et al. [28] used GMP-grade human autologous ASCs, combined with β-tricalcium phosphate (β-TCP) and BMP-2, to reconstruct a large maxillary defect resulting from the removal of a keratocyst. More recently, the same researchers successfully showed that autologous hASCs combined with β-TCP granules were able to repair large cranial defects in four humans [30]. Another clinical report demonstrated the use of ASCs to treat a large bone defect at the mandibular symphysis [31]. This case reported methods similar to those of Mesimaki et al. for the treatment of bone defects, but without requiring any ectopic bone formation. Moreover, ASCs are able to secrete several cytokines, including VEGF and hepatocyte growth factor (HGF), which contribute to the formation of a vascularized bone graft, a pre-requisite in the treatment of large bone defects. Previous research has already shown that hASCs have various differentiation potentials and can differentiate into other cells [17,32,33]. Several lines of research have illustrated that stimulating factors are able to promote proliferation and osteogenic differentiation of ASCs [34]. Thus, we hypothesized that PEMF is likely to enhance hASC proliferation and differentiation into osteoblasts or their progenitor cells.

To confirm our hypothesis, we sought to answer two questions: First, we determined whether the PEMF could promote hASC proliferation. Second, we investigated whether PEMF has the potential to promote osteogenic differentiation of hASCs.

Material and Methods

Isolation and culture of hASCs

The hASCs used in this experiment were extracted from donor adipose tissue (The Southern Hospital, China). The tissues were minced and digested with 0.1% collagenase type I (Gibco, USA) at 37 °C for 45 minutes with gentle agitation. Enzyme activity was neutralized with a two-fold volume of Dulbecco's modified Eagle's medium (DMEM, Gibco), and the samples were centrifuged for five minutes at 800 rbp/minute. The supernatant containing the lipid droplets was discarded, and the pellet was resuspended in DMEM culture medium with 10% of fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Cells were seeded onto a culture dish ($1\times10^4/cm^2$) (Corning, America) containing 10% FBS and 1% cyan-streptomycin double antibody and

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cultured in an incubator at 37°C under 5% CO_2. According to the rates of cell growth, we changed the culture medium every three days. When the confluency reached 70% to 80%, the cells were digested with trypsin-EDTA (0.25%) (Gibco, Grand Island, NY, USA), passaged at 1: 3 and subcultured in 60 mm culture dishes with a density of 1×10^4 cells per cm^2. According to the experimental requirements, hASCs were divided into a control group (without PEMF exposure) and an experimental group (subjected to PEMF).

PEMF treatment conditions

The cells in the control group were cultured in a conventional incubator condition. The cells in the experimental group were treated with PEMF in an incubator for two hours per day (the pulse field parameters were as follows: intensity 1 mT, frequency 50 Hz) for seven days, 14 days, and 21 days.

CCK-8 proliferation assay

The hASCs in the experimental and control groups were inoculated in 96-well culture plates at a concentration of 2,000 cells per well, and samples were taken on day 1, day 3, day 5, day 7, day 10, and day 13 from the experimental and control groups. Cell proliferation was assessed using the CCK-8 cell proliferation assay kit (Beyotime, China). Briefly, 10 µL of the CCK-8 solution was added to each well of the plate, and the absorbance OD at 450 mm was measured using a microplate reader (Multiskan FC, Thermo) after two hours of staining at 37°C.

Alkaline phosphatase (ALP) staining experiments

Two groups of hASCs were inoculated into 100 mm diameter dishes and were sampled on the seventh day. According to the instructions for the alkaline phosphatase (ALP) staining kit (azo coupling method), we discarded the medium, washed three times using PBS, used 4% paraformaldehyde to fix the cells for 10 minutes at 4°C, and washed with PBS three times. The incubation buffer (Beyotime China) was added, and the samples were incubated for 30 minutes in the dark. We examined the cells and took pictures under the microscope at 4× with the ImageJ image analysis software (Scion Corporation China) to analyze the image and calculate the positive expression in each field of view using the average optical density value.

RNA extraction and qRT-PCR detection

Total RNA was extracted from hASCs from the experimental and control groups at day 7, day 14, and day 21 using the RNA extraction kit. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined using a spectrophotometer. Using reverse transcription reagents (Beyotime, China), 1 µg of total RNA was reverse transcribed to cDNA. The expression of osteocalcin (OCN), osteopontin (OPN), collagen type I (COL-1), runt-related transcription factor 2 (RUNX-2), BMP-2, and ALP were detected by qRT-PCR. The primer sequences of osteogenic genes are shown in Table 1. PCR was performed using the SYBR Green Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA), which allowed real-time quantitative detection of the PCR products by measuring the increase in SYBR green fluorescence caused by the binding of SYBR green to double-stranded DNA. Each PCR sample had a volume of 20 µL, and the reaction conditions were as follows: pre-denaturation at 95°C for two minutes; then 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for a total of 40 cycles. GAPDH was used as the control for normalization. The relative quantity of mRNA was calculated ($2^{-\Delta\Delta Ct}$ analysis).

Table 1. Primers Used for qRT-PCR.

Gene	Sequences (5' to 3')	Tm (°C)
OPN	F-GTGATTTGCTTTTGCCTCCT	60
	R-GAGATGGGTCAGGGTTTAGC	
OCN	F-CTCACACTCCTCGCCCTATT	60
	R-CGCCTGGGTCTCTTCACTAC	
RUNX-2	F-GAGACTACTGCCGACCAC	60
	R-TACCTCTCCGAGGGCTACC	
ALP	F-ACCATTCCCACGTCTTCACATTTG	60
	R-AGACATTCTCTCGTTCACCGCC	
Collagen-1	F-AGGGCCAAGACGAAGACATC	60
	R-AGATCACGTCATCGCACAACA	
GAPDH	F-CACATGGCCTCCAAGGAGTAA	60
	R-GTACATGACAAGGTGCGGCTC	

Western blotting of OPN, OCN, and RUNX-2

The proteins from hASCs from the experimental and control groups at day 7, day 14, and day 21 were extracted using the cell protein extraction kit. The cells in each dish were lysed in 100 mL lysis buffer (CWBIO, Beijing, China) containing 1 mM phosphorylated protease inhibitor (Thermo, USA), and the samples were incubated on ice for 20 minutes. The extracted proteins were detected by BCA analysis and were loaded on 10% or 12% SDS-PAGE gel electrophoresis. The samples were transferred to a PVDF membrane and incubated with blocking solution of TBST containing 0.05 g/mL bovine serum albumin (BSA) for one hour. After blocking, the samples were incubated with their corresponding primary antibodies overnight at 4°C. The primary antibodies included OPN (1: 2,000, Abcam), OCN (1: 1,000, Abcam), and RUNX-2 (1: 1,000, Abcam). After washing the membrane three times in TBST, the membrane was incubated with the corresponding secondary antibody for

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one hour. After washing the membrane three times in TBST, the immunoreactive band was detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The optical densities of the bands were quantified using ImageJ (Scion Corporation).

Immunofluorescence staining of OPN, OCN, and RUNX-2

The hASCs were seeded on coverslips in plates, and the cells in the experimental and control groups that seeded on these coverslips were extracted on day 7, day 14, and day 21 to perform immunofluorescence staining. The media was aspirated from the plates, and the samples were washed twice with TBST. The hASCs were fixed with 4% formalin in PBS, pH 7.4 for 10 minutes at room temperature, followed by 0.1% Triton X-100 to rupture the membranes. Potential sites for nonspecific antibody binding were blocked with 2% BSA for one hour in PBST, pH 7.4. The cells were then incubated with OPN, OCN, or RUNX-2 polyclonal primary antibodies (1: 50 dilution, Abcam) overnight at 4°C. After washing with TBST, the samples were incubated with the HRP conjugated IgG secondary antibody (1: 100) for one hour at room temperature. The 4,6-diamidino-2-phenylindole (DAPI, Sigma) nuclear stain was added at 1 μg/mL, and the samples were incubated for 20 minutes. Cells were washed and examined under a fluorescence microscope at 10×, and images were taken.

Statistical methods

Statistical analyses were performed using SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA). The data were examined for normal distribution using Student's *t*-test. The experimental data were expressed as the mean ± standard deviation. The data were considered statistically significant when the *p* value was less than 0.05.

Results

PEMF promoted cell proliferation in hASCs at an early stage but did not have a positive effect at later stages

To explore the effect of PEMF on cell proliferation, CCK-8 assays were used to detect proliferation in hASCs (Figure 1). We directly observed the rate of cell proliferation using CCK-8. The OD value in the hASCs in the experimental group was significantly higher at day 1, day 3, day 5, and day 7 after PEMF exposure, but the OD value of hASCs in the experimental group was no longer higher or even below that of the control group after one week of PEMF exposure. These results indicated that PEMF promoted cell proliferation of hASCs at an early stage and may have a negative effect on cellular proliferation in the later period.

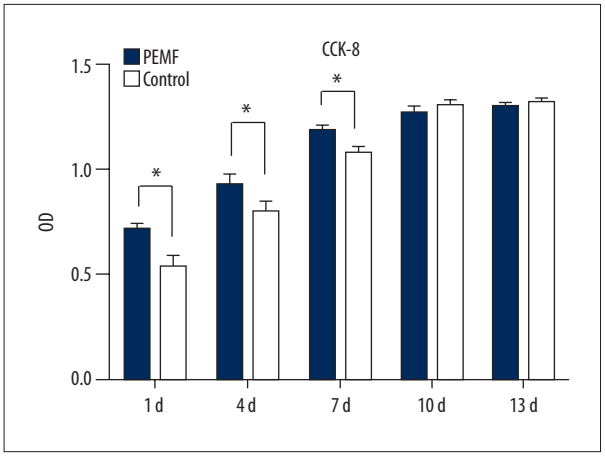

Figure 1. The growth of hASCs was measured by CCK-8 assay after 1d, 4d, 7d, 10d, and 13d. PEMF promoted the proliferation of hASCs in the experimental group compared with the control group after 1d, 4d, and 7d (* $p<0.05$). hASCs – human adipose-derived stem cells; d – day; PEMF – pulsed electromagnetic field.

PEMF had a significant effect on promoting osteogenic differentiation in hASCs

We examined the effect of PEMF on promoting osteogenic differentiation from several aspects. ALP staining was observed in the experimental and control groups after 7 days of PEMF exposure (Figure 2A, 2B). The positive staining in the experimental group was more obvious than that in the control group. Semi-quantitative analysis was performed using ImageJ Pro Plus6.0. The cumulative optical density per unit area (IOD/area) of the experimental group was significantly higher than that of the control group (Figure 2C). The qRT-PCR results after RNA extraction from the experimental and control groups at day 7, day 14, and day 21 are shown in Figure 2D–2F. The results showed that the expression of the bone-related osteogenic gene, ALP, in the experimental group was 4.3 times higher than that of the control group after a seven-day treatment. The expression of the hASC-related osteogenic gene, BMP-2, was 1.9 times higher in the experimental group than in the control group. The expression of RUNX-2 was 1.8 times higher in the experimental group than in the control group. After 14 days of treatment, RUNX-2, ALP, OPN, and OCN expression levels were 4.2, 6.8, 2.6, and 3.1 times higher, respectively, in the experimental group compared to those in the control group. At 21 days, only the expression of COL-1 was significantly increased in the experimental group (6.7-fold) compared to the control group. The results from the western blotting experiments in the experimental and control groups at day 7, day 14, and day 21 are shown in Figure 3. The results showed that the protein expression of OPN and OCN did not change significantly after seven days and 14 days after PEMF exposure, but the expression was significantly higher than that of the

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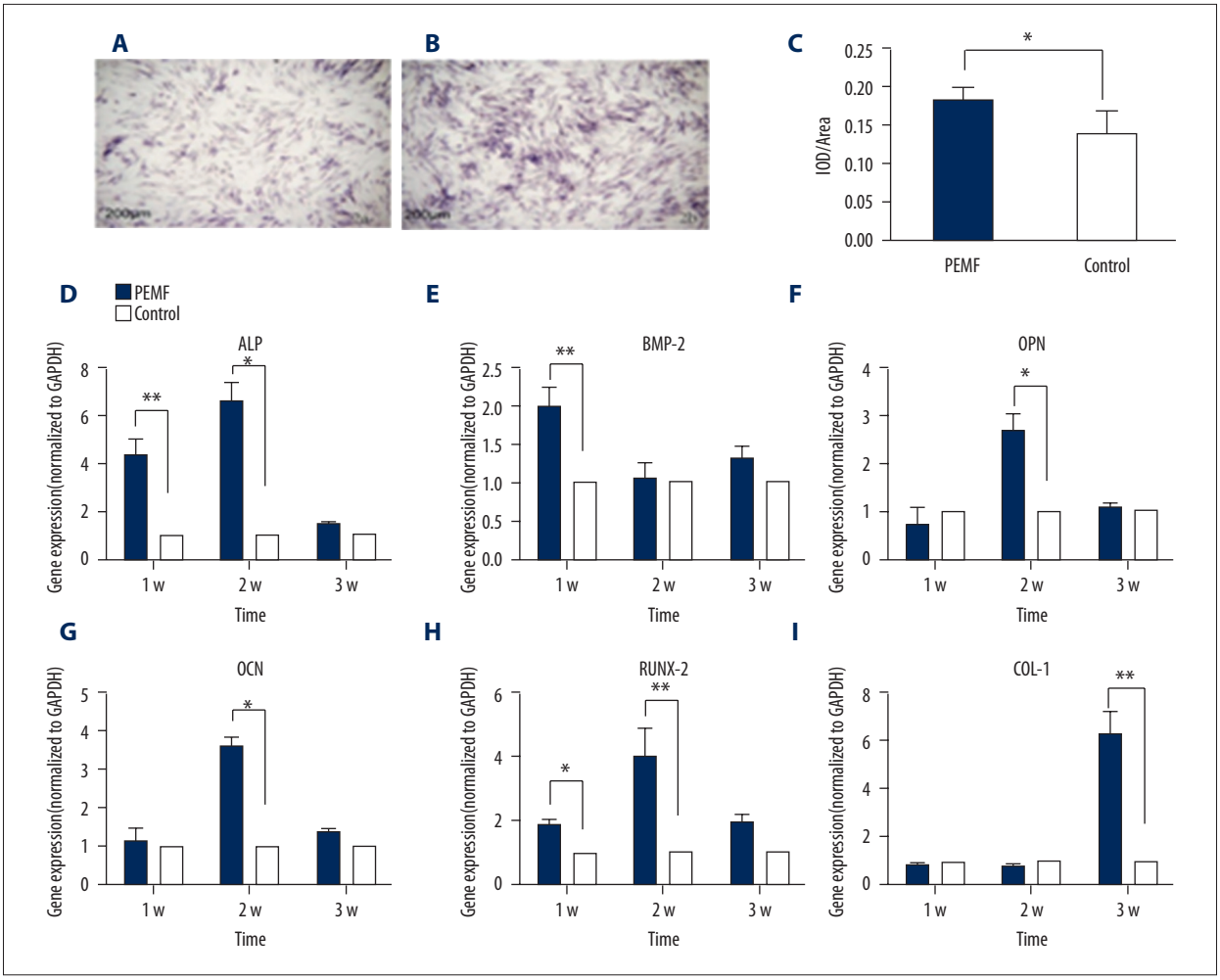

Figure 2. ALP staining and semi-quantitative analysis of hASCs; (**A**) Control group for 1w; (**B**) Experimental group induced with PEMF for 1w; (**C**) Semi-quantitative analysis of ALP staining (*$p < 0.05$). (**D**) qRT-PCR results of hASCs in the experimental and control groups after 1w, 2w, 3w. 2d: The expression of the ALP genes increased after 1w (** $p<0.01$) and 2w (* $p<0.05$) treatment with PEMF. (**E**) The expression of the BMP-2 genes increased after a 1w (** $p<0.01$) treatment with PEMF. (**F**) The expression of the OPN gene increased after a 2w (* $p<0.05$) treatment with PEMF. (**G**) The expression of the OCN gene increased after a 2w (* $p<0.05$) treatment with PEMF. (**H**) RUNX-2 genes increased after 1w (* $p<0.05$) and 2w (** $p<0.01$) treatment with PEMF. (**I**) COL-1 genes increased after a 3w (* $p<0.01$) treatment with PEMF. ALP – alkaline phosphatase; hASCs – human adipose-derived stem cells; PEMF – pulsed electromagnetic field; w – week; BMP-2 – bone morphogenetic protein-2; OCN – osteocalcin; OPN – osteopontin; COL-1 – collagen type I; RUNX-2 – runt-related transcription factor 2.

control group at 21 days. The expression of the Runx-2 protein in the experimental group was significantly higher than that in the control group at 14 days and 21 days. The immunofluorescence staining results in the experimental and control groups at day 7, day 14, and day 21 are shown in Figure 4. The fluorescence intensity of RUNX-2 in the experimental group increased at 14 days and was significantly higher than that in the control group at 21 days. However, on visual examination, the increasing trend of OPN and OCN expression in the experimental group was not obvious when compared with the expression pattern in the control group. Based on these findings, we concluded that PEMF has a significant effect on promoting osteogenic differentiation in hASCs.

Combined with these two conclusions, we found that PEMF promoted cell proliferation in hASCs at an early stage and had a relatively clear effect in promoting osteogenic differentiation of hASCs in the middle and later stages.

Discussion

Electromagnetic fields have been widely used to treat a variety of diseases [35,36]. Many researchers have shown that electromagnetic fields play an important role in both organisms and cells cultured *in vitro* [37]. In several studies, PEMF had an inhibitory effect on cell proliferation, as described by

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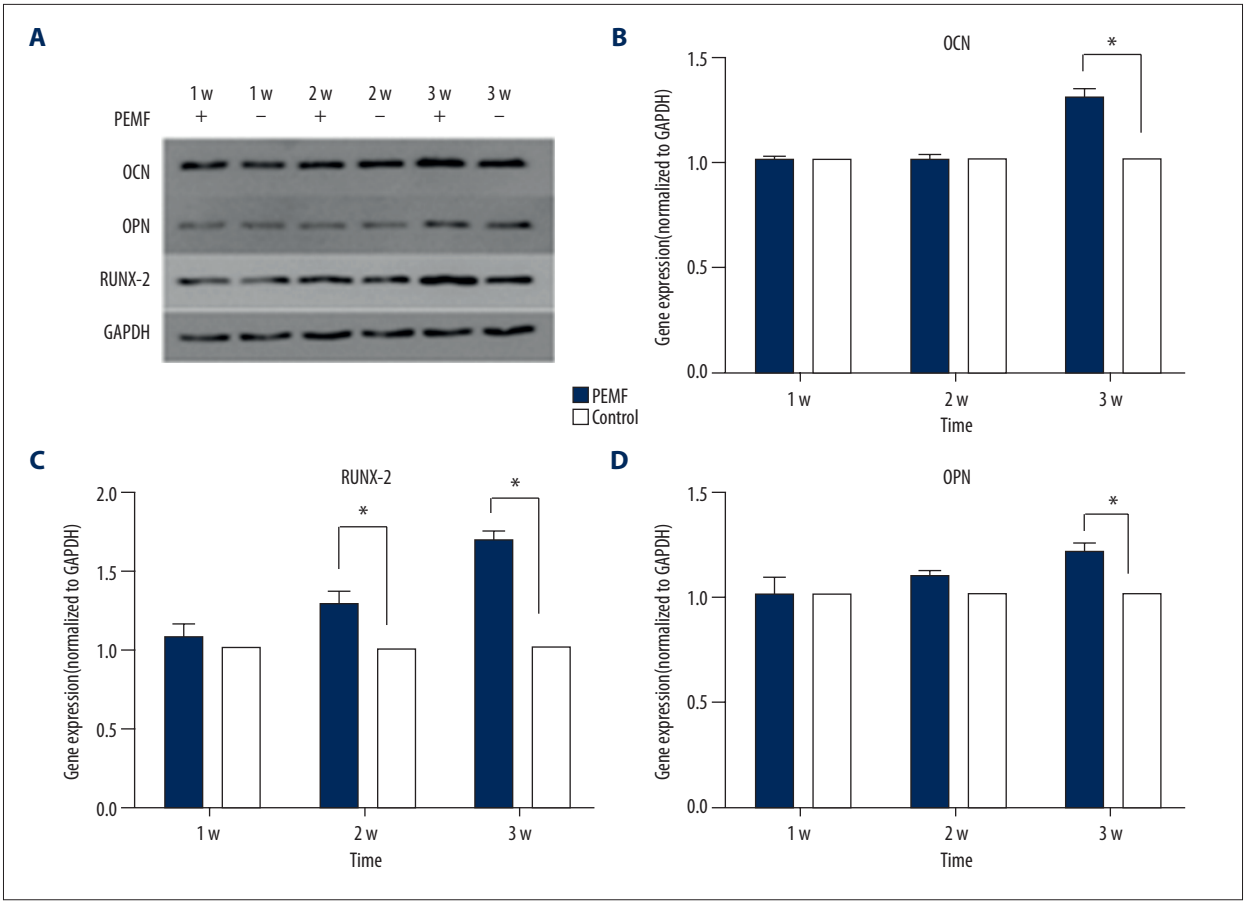

Figure 3. Western blotting of OPN, OCN, RUNX-2, and semi-quantitative analysis of hASCs in the experimental and control groups for 1w, 2w, and 3w. (**A**) Western blotting results for OPN, OCN, and RUNX-2. (**B**) Western blotting analyses of OCN. The expression of the OCN protein increased when induced with PEMF after 3w (* $p<0.05$); (**C**) Western blot analyses of RUNX-2. The expression of the RUNX-2 protein increased when induced with PEMF after 2w (* $p<0.05$) and 3w (* $p<0.05$). (**D**) Western blot analyses of OPN. The expression of the OPN protein increased when induced by PEMF after 2w (* $p<0.05$) and 3w (* $p<0.05$). OPN – osteopontin; OCN – osteocalcin; RUNX-2 – runt-related transcription factor 2; hASCs – human adipose-derived stem cells; PEMF – pulsed electromagnetic field; w – week.

Yan et al., and 20 mT PEMF exposure for 12 hours/day can inhibit the proliferation of hMSCs derived from the bone marrow [38,39]. Many studies have found that PEMF can have an effect on the biological system and can interfere with cell proliferation, morphology, apoptosis, cell differentiation, and other biological functions [6,40]. The mechanism of this effect is still not clear.

ASCs are being vigorously studies in the laboratory now, but a few clinical trials of ASCs have been reported compared to trials of BMSCs. The official clinical trial website (*https://clinicaltrials.gov/*, keyword: adipose derived stem cells) revealed 125 stem cell studies, excluding unknown status. Among them, 35 trials have been completed. Two are ongoing phase IV clinical trials [26]. In the scientific literature, ASCs have successfully been combined with biomaterials, such as β-tricalcium phosphate (β-TCP), bioactive glass (BAG), and platelet-rich plasma (PRP), to improve bone regeneration in animal models. Such studies on animals, together with several case reports on humans, provided interesting results in the field of bone regeneration [28]. Additionally, two clinical trials on bone healing have illustrated that ASCs in combination with synthetic bone graft and biomaterials may affect the regeneration, augmentation, and vascularization of large bone defects [41]. These results describe a new approach to bone tissue regeneration, based on ASCs/scaffold constructs. The use of ASC as a clinical tool has progressed significantly over the past decade. While many clinical applications of ASCs still make use of the traditional mesenchymal differentiation and proliferation principles, the concept of ASCs affecting neighboring cells via cytokine release or other paracrine mechanisms (the "bystander effect") is rapidly broadening the therapeutic potential of ASCs as a cell therapy. As a consequence, current ASC research spans the bench-to-bedside journey [42].

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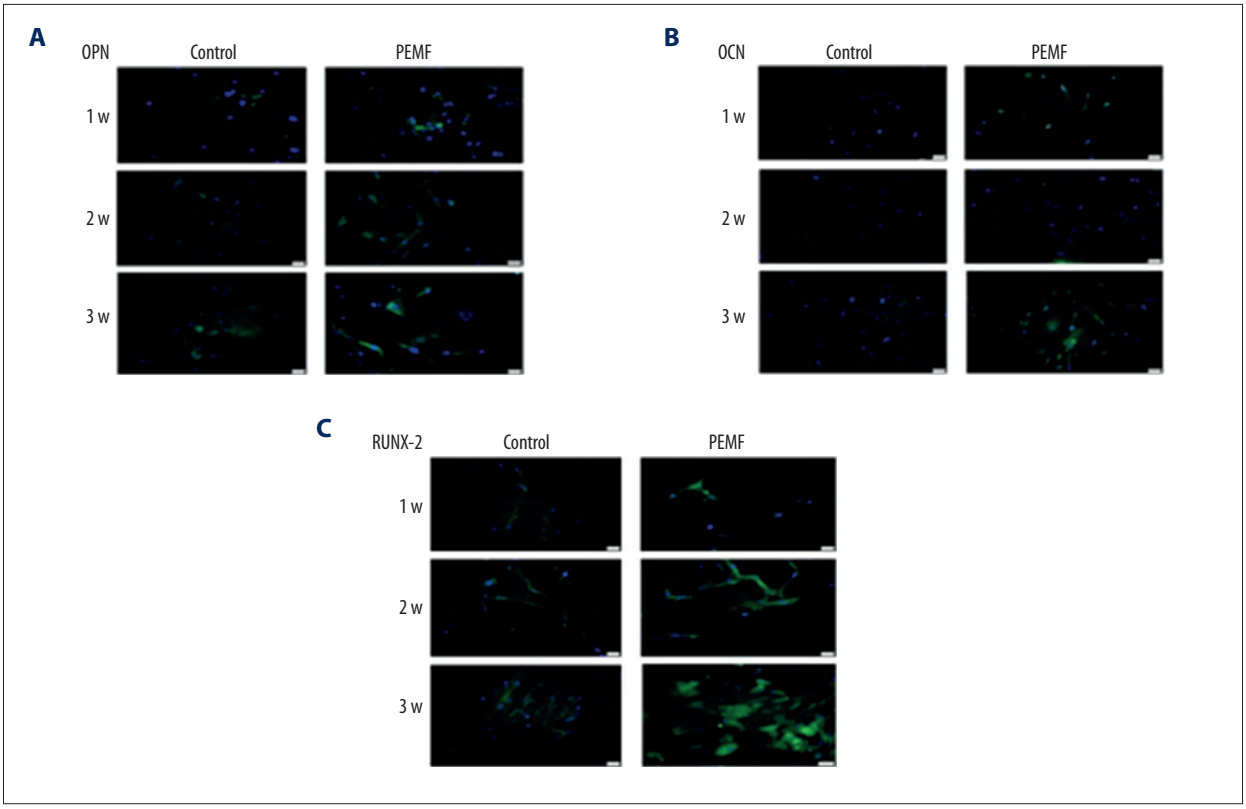

Figure 4. IF staining of OPN, OCN, RUNX-2 hASCs in the experimental and control groups for 1w, 2w, and 3w. (**A**) IF staining of OPN. the protein expression of OPN did not change significantly after 1w, 2w, and 3w. (**B**) IF staining of OCN. the expression of OCN in the experimental group was significantly higher than that of the control group at 3w while there is no obvious difference at 1w and 2w between two groups. (**C**) IF staining of RUNX-2. The expression of the Runx-2 protein in the experimental group was significantly higher than that in the control group at 2w and 3w. IF – immunofluorescence; OPN – osteopontin; OCN – osteocalcin; RUNX-2 – runt-related transcription factor 2; hASCs – human adipose-derived stem cells; w – week.

Cell proliferation is a very complex process that is controlled by a variety of cellular signal transduction pathways. Maintaining the integrity of genetic information during cell proliferation is the basis of life [34,43]. In fact, PEMF is thought to stimulate the activity of Ca^{2+} channels in the cell membrane [44]. Ca^{2+} inflow through these channels plays key roles, such as differentiation and proliferation, in the expression of specific genes that affect cell function [45]. Bekhite et al. showed that the expression of MF-mediated intracellular ROS production and Ca^{2+} as a second messenger in signaling pathways led to cardiomyocyte differentiation [46]. In one study, the extracellular Na^{+}/K^{+} concentration and osmotic pressure were also increased after ELF-EMF exposure [47]. Thus, we speculate that these findings may induce cell proliferation under PEMF exposure.

Although their application in tissue engineering and regenerative medicine is promising, the underlying mechanisms governing ASCs remain unclear. Several possible mechanisms have been proposed to address the proliferation and differentiation characteristics of ASCs [26,48]. The current study in stem cells shows that the regeneration of human tissue and the complementation of mature functional cells are due to stem cell proliferation and differentiation [49]. How exposure to PEMF *in vitro* promotes bone formation is still unclear. Electron stimulation induces a net Ca^{2+} influx in human osteoblast-like cells [50]. According to Pavalko's diffusion control/solid-state signaling model, the increase in cytosolic Ca^{2+} concentration is the starting point for signaling pathways that target specific bone matrix genes [51]. Runx-2 plays an important role in the maturation of osteoblasts by binding to its target promoters and enhancers of various bone-specific target genes through their common homologous domains [52,53]. OPN (osteopontin) is known to play an important role in the calcification of cell attachment and mineralized tissue, and OCN (osteocalcin) was recently identified to be a secreted ECM protein [54,55].

There are some limitations to our experiments. Although PEMF can promote the proliferation and differentiation of hASCs, its specific intracellular and molecular mechanisms and cell signaling pathways are not clear. Based on the conclusions of the previous experiments with special magnetic field frequencies and field strengths, we verified our hypothesis; however, the

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effects of other frequencies and field strengths on ASCs have not been explored.

Conclusions

These results suggest that PEMF can significantly promote the proliferation and osteogenic differentiation of adipose-derived stem cells so as to achieve the purpose of treating fractures and large bone defects. hASCs also have the same osteogenic differentiation potential as BMSCs. These findings can provide insight into the development of PEMF as an effective technique for regenerative medicine.

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